

AD_____

Award Number: DAMD17-02-1-0391

TITLE: Matriptase Activation in Breast Cancer Progression

PRINCIPAL INVESTIGATOR: Chen-Yong Lin, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

REPORT DATE: June 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

BEST AVAILABLE COPY

20041101 115

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE June 2004		3. REPORT TYPE AND DATES COVERED Annual (1 Jun 2003 - 31 May 2004)
4. TITLE AND SUBTITLE Matriptase Activation in Breast Cancer Progression			5. FUNDING NUMBERS DAMD17-02-1-0391	
6. AUTHOR(S) Chen-Yong Lin, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Washington, DC 20007 E-Mail: lincy@georgetown.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Original contains color plates: All DTIC reproductions will be in black and white.				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) In the current research plan, we proposed to study the mechanism for activation of matriptase, a membrane-bound serine protease. Previously, we showed matriptase is activated via transactivation, where the interactions among latent matriptase molecules, HAI-1, and other unidentified proteins are required for the proceeding of activation cleavage. In non-transformed mammary epithelial cells, matriptase activation can be induced by sphingosine 1-phosphate (S1P), a blood-borne bioactive phospholipid. In the past one-year, we further showed that S1P induces matriptase translocation and accumulation at cell-cell contacts where activation occurs. We further showed that both matriptase translocation and activation depend on the assembly of adherens junctions and formation of subcortical actin belts in response to S1P exposure. Disruption of subcortical actin belt formation and prevention of adherens junction assembly led to prevention of accumulation and activation of the protease at cell-cell contacts. The coupling of matriptase activation to adherens junction assembly and actin cytoskeletal rearrangement may serve to ensure tight control of matriptase activity, restricted to cell-cell junctions of mammary epithelial cells.				
14. SUBJECT TERMS No Subject Terms Provided.				15. NUMBER OF PAGES 18
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified
				20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

Cover.....	
SF 298.....	1
Table of Contents.....	2
Introduction.....	3
Body.....	3
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusions.....	6
References.....	6
Appendices.....	6

Introduction:

Invasion of cells from one tissue to a neighboring tissue has been observed both in physiological processes, such as organ development and wound healing, and pathological processes, such as breast cancer metastases. Physiological invasion is thought to be tightly controlled. The regulatory mechanism of physiological invasion may be lost in pathologic states, particularly in malignant progression, resulting in uncontrolled invasion. Degradation of extracellular matrix (ECM) and cell migration are two key events for cellular invasion. Proteases and protease inhibitors are implicated in cellular invasion due to their potential roles in localized degradation of ECM and in the activation of latent growth/motility/angiogenesis factors, such as hepatocyte growth factor (HGF)/scatter factor (SF). HGF/SF is a major regulatory molecule for epithelial cell migration. HGF/SF and most ECM-degrading protease systems, such as the uPA system and the matrix metalloproteases are, however, expressed *in vivo* by the stromal elements of human breast cancer. Therefore, breast cancer cell invasion has been proposed to be a collaboration between epithelial cancer cells and stromal cells. In order to understand the role of epithelial cells in cellular invasion, we have characterized an epithelial-derived, breast cancer-associated, integral membrane, trypsin-like serine protease, termed matriptase (1). Matriptase has been demonstrated to activate uPA and HGF/SF (2). These observations suggest that matriptase could act as a cell surface activator to recruit and activate stromal-derived ECM-degrading proteases and motility factors. Furthermore, activation of matriptase in nontumorigenic human mammary epithelial cells (HMEC) seems to be tightly regulated by bioactive lipids, mainly sphingosine 1-phosphate (S1P) (3,4). Surprisingly, this S1P-based, physiological regulatory mechanism may be lost during breast cancer progression (5). In the current research plan, we proposed to study the role of S1P receptors in matriptase activation and the structural requirements for the activation of matriptase.

Body:

During the June 03-May 04 period, we had addressed Aim 1 of the original proposal. We have produced mAb directed against EDG 1 receptor and in the process to produce mAbs against EDG 3 and EDG 5. The mechanistic study on how sphingosine 1-phosphate (S1P) induces matriptase activation in mammary epithelial cell went very well in the past year. These works have been published in American Journal Physiology-Cell Physiology (286: C1159-C1169, 2004). We report here with these works. Please refer to the attached reprint for the Figures.

Aim 1: To investigate how sphingosine 1-phosphate (SPP) induces activation of matriptase in non-malignant mammary epithelial cells.

Year 2: We will finish expression analyses of EDG receptors in mammary epithelial cells and breast cancer cells in Aim 1 and begin to prepare EDG receptor proteins as immunogens to produce antibodies directed against these EDG receptors.

S1P induces accumulation of matriptase at cell-cell contacts-- In our previous studies (1;2), sphingosine 1-phosphate (S1P), a lysophospholipid present in serum, was identified as a blood-borne, non-protease factor that induces the activation of matriptase in immortal human mammary epithelial cells. S1P also simultaneously causes profound changes in cell morphology, including cell shape changes, increases in cell-cell contacts, and cell spreading. These S1P-induced morphological changes are mainly associated with actin cytoskeleton rearrangement. Formation of F-actin at cell peripheries (subcortical actin belt) is the most striking change in 184 A1N4 cells. Because direct interaction between S1P and matriptase is not the mechanism for S1P-induced matriptase activation, we reasoned that matriptase activation could be associated with these S1P-induced cellular events and/or signal transduction (1). In the current study, we first examined the subcellular localization of matriptase during the process of its activation. After growth in IMEM- supplemented with 0.5% serum for 2 days, matriptase (as the latent form) was observed at cell-cell contacts as well as within cells (Figs. 1a and 2b; in the attached paper). A relatively low level of F-actin was also observed at cell-cell borders and within cells (Fig. 1b). Ten min after exposure to S1P, matriptase increased at cell-cell contacts (Figs. 1d and 2e), suggesting that S1P induces accumulation of matriptase at cell-cell contacts. In spite of accumulation of matriptase at cell-cell contacts, matriptase was observed at very low levels or was absent at the peripheries of

cells without contact with neighboring cells (Fig. 1d). Figure 1g shows that matriptase is absent on the extended, leading edge ruffle of an isolated cell, but matriptase was seen at small contact sites on cell peripheries touched by long cellular processes of other cells (Figs. 1g and 1i). These results suggest that cell-cell contact may be required for matriptase to stably reside on the cell surface. A parallel change in actin cytoskeletal structure was also observed in response to S1P treatment (Figs. 1b, 1e, 1h, 3b, and 3e). Formation and accumulation of actin filaments were observed at cell peripheries, mainly at cell-cell contacts. The localization of matriptase at cell-cell contacts closely coincided with that of F-actin (Figs. 1c, 1f, and 1i).

The kinetics of matriptase activation at cell-cell contacts-- We further examined the appearance and the localization of activated matriptase relative to total matriptase (Fig. 2) and to F-actin (Fig. 3) in 184 A1N4 cells at different time intervals, following exposure to S1P. After growth in 0.5% serum for two days, 184 A1N4 cells do not express activated matriptase (1-3). Immunofluorescence, using mAb M69 that recognizes two-chain matriptase, showed only non-specific staining, with weak diffuse staining in the cytosol (Fig. 2a), as seen in the control with FITC-labeled secondary antibody alone (data not shown). Ten min after exposure to S1P, activated matriptase was detected mainly as tiny spots (Fig. 2d) and the total matriptase accumulated at cell-cell contacts (Fig. 2e). When both images were merged, activated matriptase was clearly colocalized with total matriptase at cell-cell contacts. At 20 and 30 min, activated matriptase was detected more brightly and in elongated patterns (Figs. 3a and 3d). When comparing the localization of activated matriptase with the newly formed F-actin at the cell peripheries, activated matriptase closely coincided with F-actin at cell-cell contacts (Figs. 3c and 3f). We also noticed that subcortical actin belts were not homogeneously formed around the periphery of cells. More F-actin accumulated at the edges of cell-cell contacts (Figs. 3b and 3e). Interestingly, activated matriptase was detected right at those areas where F-actin accumulated the most (Figs. 3c and 3f). At 30 min, the staining of activated matriptase elongated along cell-cell contacts (Fig. 3d), and more F-actin accumulated at the cell peripheries (Fig. 3e). Additionally, activated matriptase, like total matriptase, was only detected at sites of cell-cell contacts, for example in cell clusters (Figs. 2 and 3) or between two cells (Fig. 3g).

S1P induces assembly of adherens junctions in mammary epithelial cells— Because formation of subcortical actin belts at adherens junctions is induced by E-cadherin-mediated cell-cell contact formation (4), and because the role of S1P in adherens junction assembly has been well documented in endothelial cells (5), we further investigated whether S1P also can induce adherens junction assembly in mammary epithelial cells and whether the S1P-induced accumulation of matriptase at cell-cell contacts depends on adherens junction assembly. Given that extracellular Ca^{++} is required for the homotypic binding between cadherin molecules on adjacent cells, we modulated the availability of extracellular Ca^{++} , in combination with S1P, to investigate whether S1P can induce assembly of adherens junctions. 184 A1N4 cells were first cultured in regular, calcium-containing (1.8 mM) medium, supplemented with 0.5% serum for two days. After switching the cells to calcium-free medium for 30 min, E-cadherin was detected as diffuse pattern in cytosol, and the subcortical actin belt was not observed (Fig. 4A). By increasing extracellular Ca^{++} to 1000 μM , in the absence of S1P, the subcortical actin belt and E-cadherin began to appear at the sites of cell-cell contact, but in very low levels (Fig. 4A). We next exposed these cells to S1P for 30 min. In the absence of extracellular Ca^{++} , S1P caused some rounding of cells (Fig. 4B). Likewise, there was no translocation of E-cadherin to cell-cell contacts, nor formation of subcortical actin belts (Fig. 4B). By increasing extracellular Ca^{++} to 100 and 1,000 μM , S1P induced to a greater extent, translocation of E-cadherin to cell-cell contacts and the formation of subcortical actin belts (Fig. 4B). While low levels of E-cadherin were seen at the cell-cell contacts when 184 A1N4 cells were grown in media containing adequate extracellular Ca^{++} , in the absence of S1P, these cell-cell adhesion E-cadherin failed to resist the extraction of cells with a cytoskeletal stabilizing buffer containing 0.5% Triton X-100 (Fig. 5). In contrast, S1P induced assembly of strong adherens junctions, which were resistant to a wash with 0.5% Triton X-100 (Fig. 5). These data suggest that S1P can induce adherens junction assembly in mammary epithelial cells, as it does in endothelial cells. In contrast to E-cadherin, although matriptase accumulated at cell-cell contacts in response to S1P treatment, the protease was washed away by 0.5% Triton X-100 (Fig. 5). These observations suggest that while matriptase localization was coincident with that of E-cadherin, at cell-cell contacts, the protease may not be incorporated into the tightly, bound E-cadherin plaques.

Adherens junction assembly and subcortical actin belt formation are required for matriptase accumulation and activation at cell-cell contacts— The concurrence of matriptase accumulation and activation at cell-cell contacts, adherens junction assembly, and subcortical actin belt formation suggests a potential functional relationship between both S1P-induced events. Therefore, we further investigated whether prevention of subcortical actin belt formation, by disruption of F-actin polymerization, and prevention of adherens junction assembly, by preventing homotypic binding of E-cadherin, each affect the accumulation and activation of matriptase. The pharmacological agents latrunculin B and cytochalasin D, (which can inhibit actin polymerization and disrupt microfilament organization), abolished S1P-induced activation of matriptase (Fig. 6). Both latrunculin B (Fig. 6, lanes 2 and 3) and cytochalasin D (Fig. 6, lane 7), but not nocodazole (Fig. 6, lane 5), (an inhibitor of microtubule polymerization), completely inhibited S1P-induced matriptase activation. Immunofluorescent staining further revealed that cytochalasin D (Fig. 7) and latrunculin B (data not shown) prevented not only formation of subcortical actin belt and assembly of adherens junction, but also accumulation of matriptase to cell peripheries.

We further modulated the availability of extracellular Ca^{++} , in combination with S1P, to examine the effects of adherens junction assembly on matriptase accumulation at cell-cell contacts. When 184 A1N4 cells were cultured in medium containing 10 μM Ca^{++} , S1P failed to induce translocation and accumulation of matriptase at cell-cell contacts, nor β -catenin, an adherens junction marker protein (Fig. 8). By increasing extracellular Ca^{++} to 1000 μM , in the absence of S1P, low levels of matriptase and β -catenin were observed at cell-cell contacts. When exposing these cells to S1P, both matriptase and β -catenin significantly accumulated at cell-cell contacts (Fig. 8). In addition to preventing the accumulation of matriptase to cell-cell contacts, removal of extracellular Ca^{++} was showed to abrogate S1P-induced matriptase activation (1). These data suggest that S1P-induced assembly of adherens junctions is required for S1P-induced accumulation and activation of matriptase at cell-cell contacts.

Monoclonal antibody M32, which is directed against matriptase LDLR class A domain III, inhibits S1P-induced matriptase activation, but not S1P-induced actin cytoskeletal rearrangement-- In our previous study, the intact LDLR class A domains of matriptase were shown to be required for matriptase activation in breast cancer cells (6). This observation is consistent with the hypothesis that matriptase activation is carried out by transactivation, which could require complex protein-protein interactions among matriptase zymogens and other unidentified molecules. Alterations at the calcium cages in LDLR class A domains of matriptase by site-directed point mutation abolished matriptase activation in breast cancer cells (6). Interestingly, this alteration in LDLR class A domain III also destroyed the epitope recognized by the anti-matriptase mAb M32 (Fig. 9). Therefore, we further tested whether this anti-matriptase mAb could inhibit S1P-induced matriptase activation and S1P-induced actin cytoskeletal rearrangement in immortal mammary epithelial cells. Pretreatment of 184 A1N4 cells with mAb M32 for 1 hr clearly showed inhibition of S1P-induced matriptase activation (Fig. 10, g). This inhibition of S1P-induced matriptase activation by mAb M32 could result from the potential inhibitory activity of matriptase transactivation. Alternatively, pretreatment of mAb M32, which could bind to the matriptase on the cell surfaces before S1P treatment (Figs. 1a, 2b, and 8), could cause internalization of matriptase, and thus may interfere with its later accumulation induced by S1P treatment, as matriptase was seen as punctate, following S1P treatment (Fig. 10, d). In contrast to the inhibition of matriptase activation, anti-matriptase mAb M32 did not affect the formation of subcortical actin belts (Fig. 10, e and h). These results suggest that while actin cytoskeletal rearrangement is required for S1P-induced matriptase activation, matriptase activity is not important for S1P-induced actin cytoskeletal rearrangement.

Key research accomplishments:

We showed that S1P induces matriptase translocation to cell-cell contacts where activation occurs. We further showed that both matriptase translocation and activation depend on the assembly of adherens junctions and formation of subcortical actin belts in response to S1P exposure. Disruption of subcortical actin belt formation and prevention of adherens junction assembly led to prevention of accumulation and activation of the protease at cell-cell contacts. We also used mAb M32, directed against one of the LDL receptor class A domains of matriptase, to block S1P-induced activation of matriptase.

Reportable outcomes:

1. Oberst, M.D., Singh, B., Ozdemirli, M., Dickson, R.B., Johnson, M.D., and Lin, C.-Y. (2003) Characterization of Matriptase Expression in Normal Human Tissues. *J. Histochem. and Cytochem.* 51:1017-1025
2. Oberst, M.D., Williams, C., Johnson, M.D., Dickson, R.B., and Lin, C.-Y. (2003) The activation of matriptase requires its noncatalytic domains, serine protease domain, and its cognate inhibitor. *J. Biol. Chem.* 278:26773-26779.
3. Hung RJ, Hsu I. W., Dreiling J. L., Lee M. J., Williams C. A., oberst M. D., Dickson R. B., and Lin C.-Y. (2004) Assembly of Adherens Junctions Is Required for Sphingosine 1-phosphate-induced Matriptase Accumulation And Activation at Mammary Epithelial Cell-cell Contacts. *Am J Physiol Cell Physiol.* 286:C1159-69.

Conclusion:

The activation of matriptase requires proteolytic cleavage at a canonical activation motif that converts the enzyme from a one-chain zymogen to an active, two-chain protease. This activating cleavage occurs via a transactivation mechanism where interaction between matriptase zymogen molecules leads to activation of the protease. Accumulation of matriptase at cell-cell contacts apparently provides required physical contacts for matriptase zymogens and other required proteins for the proceeding of matriptase activation. The coupling of matriptase activation to adherens junction assembly and actin cytoskeletal rearrangement may serve to ensure tight control of matriptase activity, restricted to cell-cell junctions of mammary epithelial cells.

Reference:

1. Benaud, C., Oberst, M., Hobson, J.P., Spiegel, S., Dickson, R.B., and Lin, C.Y. (2002) *J Biol. Chem.* 277, 10539-10546.
2. Benaud, C.M., Oberst, M., Dickson, R.B., and Lin, C.Y. (2002) *Clin Exp. Metastasis* 19, 639-649.
3. Benaud, C., Dickson, R.B., and Lin, C.Y. (2001) *Eur. J Biochem.* 268, 1439-1447.
4. Adams, C.L., Chen, Y.T., Smith, S.J., and Nelson, W.J. (1998) *J Cell Biol.* 142, 1105-1119.
5. Lee, M.J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I., and Hla, T. (1999) *Cell* 99, 301-312.
6. Oberst, M.D., Williams, C.A., Dickson, R.B., Johnson, M.D., and Lin, C.Y. (2003) *J Biol. Chem.* 278, 26773-26779.

Appendices:

A reprint of the following paper is provided:

Hung RJ, Hsu I. W., Dreiling J. L., Lee M. J., Williams C. A., oberst M. D., Dickson R. B., and Lin C.-Y. (2004) Assembly of Adherens Junctions Is Required for Sphingosine 1-phosphate-induced Matriptase Accumulation And Activation at Mammary Epithelial Cell-cell Contacts. *Am J Physiol Cell Physiol.* 286:C1159-69.

Assembly of adherens junctions is required for sphingosine 1-phosphate-induced matriptase accumulation and activation at mammary epithelial cell-cell contacts

Ruei-Jiun Hung,* Ia-Wen J. Hsu,* Jennifer L. Dreiling, Mon-Juan Lee, Cicely A. Williams, Michael D. Oberst, Robert B. Dickson, and Chen-Yong Lin

Department of Oncology, Lombardi Cancer Center, Georgetown University Medical Center, Washington, District of Columbia 20057

Submitted 18 September 2003; accepted in final form 3 December 2003

Hung, Ruei-Jiun, Ia-Wen J. Hsu, Jennifer L. Dreiling, Mon-Juan Lee, Cicely A. Williams, Michael D. Oberst, Robert B. Dickson, and Chen-Yong Lin. Assembly of adherens junctions is required for sphingosine 1-phosphate-induced matriptase accumulation and activation at mammary epithelial cell-cell contacts. *Am J Physiol Cell Physiol* 286: C1159–C1169, 2004; 10.1152/ajpcell.00400.2003.—Sphingosine 1-phosphate (S1P), a bioactive phospholipid, simultaneously induces actin cytoskeletal rearrangements and activation of matriptase, a membrane-associated serine protease in human mammary epithelial cells. In this study, we used a monoclonal antibody selective for activated, two-chain matriptase to examine the functional relationship between these two S1P-induced events. Ten minutes after exposure of 184 A1N4 mammary epithelial cells to S1P, matriptase was observed to accumulate at cell-cell contacts. Activated matriptase first began to appear as small spots at cell-cell contacts, and then its deposits elongated along cell-cell contacts. Concomitantly, S1P induced assembly of adherens junctions and subcortical actin belts. Matriptase localization was observed to be coincident with markers of adherens junctions at cell-cell contacts but likely not to be incorporated into the tightly bound adhesion plaque. Disruption of subcortical actin belt formation and prevention of adherens junction assembly led to prevention of accumulation and activation of the protease at cell-cell contacts. These data suggest that S1P-induced accumulation and activation of matriptase depend on the S1P-induced adherens junction assembly. Although MAb M32, directed against one of the low-density lipoprotein receptor class A domains of matriptase, blocked S1P-induced activation of the enzyme, the antibody had no effect on S1P-induced actin cytoskeletal rearrangement. Together, these data indicate that actin cytoskeletal rearrangement is necessary but not sufficient for S1P-induced activation of matriptase at cell-cell contacts. The coupling of matriptase activation to adherens junction assembly and actin cytoskeletal rearrangement may serve to ensure tight control of matriptase activity, restricted to cell-cell junctions of mammary epithelial cells.

serine protease; phospholipid; actin

MATRIPTASE (also known as TADG-15, ST-14, MT-SP1, and epithin), is a membrane-associated, multiple-domain serine protease that contains a short cytoplasmic domain at the amino terminus, followed by a putative transmembrane domain, a sperm protein, enterokinase and agrin (SEA) domain, two tandem C1r/s, Uegf, and bone morphogenic protein-1 (CUB) domains, four tandem low-density lipoprotein (LDL) receptor (LDLR) class A domains, and a trypsinlike serine protease

domain at the most carboxy terminus (10, 16, 27, 28, 32). Matriptase belongs to the type II transmembrane serine protease family, a rapidly expanding serine protease subfamily with a putative amino-terminal transmembrane domain (8, 19). Matriptase is expressed by epithelial elements of almost all human organs examined so far by immunohistochemistry with tissue microarrays (22). The pattern of matriptase expression in normal tissues suggests that matriptase may play a broad role in the biology of surface-lining epithelial cells. Recently, matriptase^{-/-} knockout mice showed that matriptase was essential for postnatal survival. The postnatal death of mice resulted from a deficient epidermal barrier function in the skin of newborn matriptase^{-/-} mice (18). In addition, these mice had abnormal hair follicle development and disturbed thymic homeostasis, as indicated by increased lymphocyte apoptosis in the thymus of newborn mice.

In addition to normal tissues, matriptase is also expressed in vivo by human carcinomas of various sites, including those of the breast, ovary, colon, endometrium, stomach, and prostate (20, 21, 25, 28). Because matriptase is able to activate urokinase-type plasminogen activator (uPA) and hepatocyte growth factor (HGF)/scatter factor in vitro, it has been proposed to play an important role in cancer invasion and metastasis, which require the degradation of extracellular matrix and enhanced cellular motility (14, 26). In addition, tight correlation between the expression of matriptase and its HGF substrate as well as the c-Met receptor was observed in a cohort of 330 node-negative breast carcinomas (9). More importantly, high-level expression of the c-Met receptor, matriptase, and matriptase cognate inhibitor HGF activator inhibitor 1 (HAI-1) (15) were associated with poor patient outcome in the same set of node-negative breast carcinomas (9).

Matriptase also may be deregulated at its activation in human breast cancer cells (3). Activation of matriptase, which requires cleavage at its activation motif to convert a single-chain zymogen to the two-chain active protease, is likely to be carried out by a transactivation mechanism (23), an alternative mechanism whereby latent matriptase zymogen molecules interact with each other, leading to the activation cleavage. Transactivation of serine proteases generally occurs for the serine proteases at the pinnacle of protease cascades to first generate active protease. The noncatalytic domains of matriptase, particularly its LDLR class A domains, and post-

*R.-J. Hung and I.-W. J. Hsu contributed equally to this work.

Address for reprint requests and other correspondence: C.-Y. Lin, Lombardi Cancer Center, Georgetown Univ. Medical Center 3970 Reservoir Rd. NW, Washington, DC 20057-1412 (E-mail address: lincy@georgetown.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

translational modifications, such as amino-terminal processing by cleavage at Gly149 within the SEA domain and glycosylation at the first CUB domain and at the serine protease domain, are required for matriptase activation (23). These structural requirements for matriptase activation could reflect the structural basis for protein-protein interactions among matriptase zymogens and other proteins required for transactivation of the protease (23). Once activated, HAI-1 acts to terminate active matriptase to avoid undesired proteolysis of matriptase. Subsequently, matriptase-HAI-1 complexes are shed from cell surfaces into the extracellular milieu (2). Although the details of how transactivation occurs remain unknown, deregulation of matriptase transactivation has been observed in breast cancer cells (3). In immortalized mammary epithelial cells, matriptase activation, its subsequent inhibition by HAI-1, and its clearance by shedding are all dependent on the presence of a blood-borne lipid mediator, sphingosine 1-phosphate (S1P) (2, 4). The S1P-dependent activation of matriptase is, however, lost in breast cancer cells (3).

S1P is a major lipid mediator in serum, and it possesses growth factor-like activities. S1P induces a wide spectrum of biological responses in a variety of cell types, including proliferation, survival, actin cytoskeletal rearrangement, cell shape changes, contraction, and cellular motility. These cellular responses are believed to involve family members of endothelial differentiation gene (*EDG*) receptors, a subfamily of G protein-coupled, heptahelical membrane receptors and their intracellular downstream effectors, including adenylate cyclase, Rho family GTPases, phospholipase C, protein kinase C, MAP kinase, and phosphatidylinositol 3-kinase (11, 13, 24, 29, 31). In our previous study (4), matriptase was identified as a unique extracellular effector of S1P. We also observed that S1P induces prominent actin cytoskeletal rearrangement and formation of subcortical actin belts, which are likely to be associated with assembly of adherens junctions, which are known to require extracellular Ca^{2+} and to be important for stable cell-cell adhesion. Because direct interaction of S1P with matriptase has been excluded in an extracellular Ca^{2+} -dependent activation of matriptase (4), we now propose that matriptase activation could be an indirect, downstream consequence of S1P-induced cellular responses. In this study, we investigated the functional relationship between S1P-induced actin cytoskeletal rearrangement and matriptase activation. Our results suggest that in immortalized mammary epithelial cells, S1P-induced matriptase accumulation at cell-cell contacts depends on S1P-induced adherens junction assembly and subcortical actin belt formation, a mechanism that may ensure that matriptase functions only at cell-cell contacts.

MATERIALS AND METHODS

Chemicals and reagents. 4',6-Diamidino-2-phenylindole (DAPI) was obtained from Sigma (St. Louis, MO). Formaldehyde solution was obtained from EM Science (EM Industries, Bibbstown, NJ). Latrunculin B, cytochalasin D, and nocodazole were obtained from Biomol (Plymouth Meeting, PA). All other chemical reagents were purchased from Sigma unless otherwise specified.

Cell lines and culture conditions. Immortalized 184 A1N4 human mammary epithelial cells (provided by Martha Stampfer, Lawrence Berkeley National Laboratory, Berkeley, CA) were maintained as previously described (2). BT549 human breast cancer cells were maintained in culture by growth in Iscove's minimal essential medium

(IMEM; Invitrogen, Rockville, MD) supplemented with 5% FBS in a humidified chamber at 37°C and 5% CO_2 .

Monoclonal antibodies. Matriptase protein was detected with the M32 monoclonal antibody (MAB) that recognizes both the latent (1 chain) and activated (2 chain) forms of the protease or with the M69 monoclonal antibody that recognizes an epitope present only in the activated (2 chain) form of the enzyme (2, 4). MAB M32 recognizes a 70-kDa processed form and the 120-kDa matriptase-HAI-1 complex; M32 also recognizes the 95-kDa full-length matriptase, which was barely detectable in those cells endogenously expressing the protease but becomes prominent in those cells that were used for overexpression of matriptase (23). We have also found that the epitope against which MAB M32 is directed is destroyed by a point mutation in LDLR class A domain III (see Fig. 9). Therefore, MAB M84, which is directed against the serine protease domain of matriptase, was used in Fig. 9 to show the expression of matriptase mutants. Anti-E-cadherin MAB (clone 36) and FITC-labeled anti- β -catenin MAB (clone 14) were purchased from BD Bioscience.

Labeling MABs with fluorescent dyes. To label MABs with fluorescent dyes, 1 mg each of MABs M32 and M69 were solubilized in 0.1 M sodium bicarbonate and then labeled with Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR), according to the manufacturer's instructions. The unbound Alexa Fluor dyes were removed by dialysis against PBS. The ratios of mole of dye per mole of IgG were determined to be ~2.5–3.5 for Alexa Fluor 488 and 5–6 for Alexa Fluor 594.

Constructs and transfections. The cDNA clones for the HAI-1, wild-type matriptase, or matriptase mutants, bearing point mutations at the calcium cages of LDLR class A domains (23), in the vector pcDNA3.1 (Invitrogen, Carlsbad, CA) were used in transient transfections. Transient transfection of matriptase or HAI-1 constructs was accomplished by using Eugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. When cotransfections were conducted, the amount of DNA used with the transfection reagent was kept constant for each individual transfection by including empty vector pcDNA3.1 DNA where appropriate.

Western blotting. Protein for Western blotting was prepared by the lysis of cells in 1% Triton X-100 in PBS or RIPA buffer (0.1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS in PBS), after washing cells two times in PBS. Insoluble debris was removed by centrifugation, and the protein concentration was measured with bicinchoninic acid protein assay reagents (Pierce, Rockford, IL) according to the manufacturer's protocol. Lysates were resolved by SDS-PAGE under nonboiled and nonreduced conditions and then transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were probed with MABs. The binding of the primary antibody was followed by recognition with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), and detected with the Western Lightening Chemiluminescence Reagent Plus (Perkin Elmer, Boston, MA). The MABs recognizing matriptase were generated against conformation-dependent epitopes, and therefore samples were run under nonreducing SDS-PAGE conditions and were not boiled before electrophoresis to preserve the formation of complexes between active matriptase and HAI-1.

Immunofluorescence microscopy. Cells plated onto microcover glasses were fixed in 3.7% formaldehyde in PBS for 20 min at room temperature. Cells were permeabilized by 0.05% Triton X-100 in PBS. Matriptase and activated matriptase were detected with Alexa Fluor dye-labeled M32 or M69. β -Catenin was detected by FITC-labeled anti- β -catenin MAB (clone 14; BD Bioscience, San Jose, CA). E-cadherin was detected by anti-E-cadherin MAB (clone 36; BD Bioscience). For the double labeling of matriptase and E-cadherin, E-cadherin MAB was used, followed by FITC-labeled anti-mouse IgG and then Alexa Fluor 594-conjugated MAB M32 in PBS containing

250 $\mu\text{g/ml}$ mouse IgG and 3% BSA. Actin was visualized with Texas red-conjugated phalloidin (Molecular Probes), and nuclei were visualized by DAPI staining. Cover glasses were mounted with Prolong Antifade (Molecular Probes) and observed on a Nikon Eclipse E600 digital fluorescence microscope, and images were captured with the Metavue software package for the Nikon digital microscope.

RESULTS

SIP induces accumulation of matriptase at cell-cell contacts. In our previous studies (3, 4), SIP, a lysophospholipid present in serum, was identified as a blood-borne, nonprotease factor that induces the activation of matriptase in immortalized human mammary epithelial cells. SIP also simultaneously causes profound changes in cell morphology, including cell shape changes, increases in cell-cell contacts, and cell spreading. These SIP-induced morphological changes are mainly associated with actin cytoskeleton rearrangement. Formation of F-actin at cell peripheries (subcortical actin belt) is the most striking change in 184 A1N4 cells. Because direct interaction

between SIP and matriptase is not the mechanism for SIP-induced matriptase activation, we reasoned that matriptase activation could be associated with these SIP-induced cellular events and/or signal transduction (4). In the current study, we first examined the subcellular localization of matriptase during the process of its activation. After growth in IMEM supplemented with 0.5% serum for 2 days, matriptase (as the latent form) was observed at cell-cell contacts as well as within cells (Figs. 1A and 2B). A relatively low level of F-actin was also observed at cell-cell borders and within cells (Fig. 1B). Ten minutes after exposure to SIP, matriptase increased at cell-cell contacts (Figs. 1D and 2E), suggesting that SIP induces accumulation of matriptase at cell-cell contacts. Despite accumulation of matriptase at cell-cell contacts, matriptase was observed at very low levels or was absent at the peripheries of cells without contact with neighboring cells (Fig. 1D). Figure 1G shows that matriptase is absent on the extended, leading-edge ruffle of an isolated cell, but matriptase was seen at small

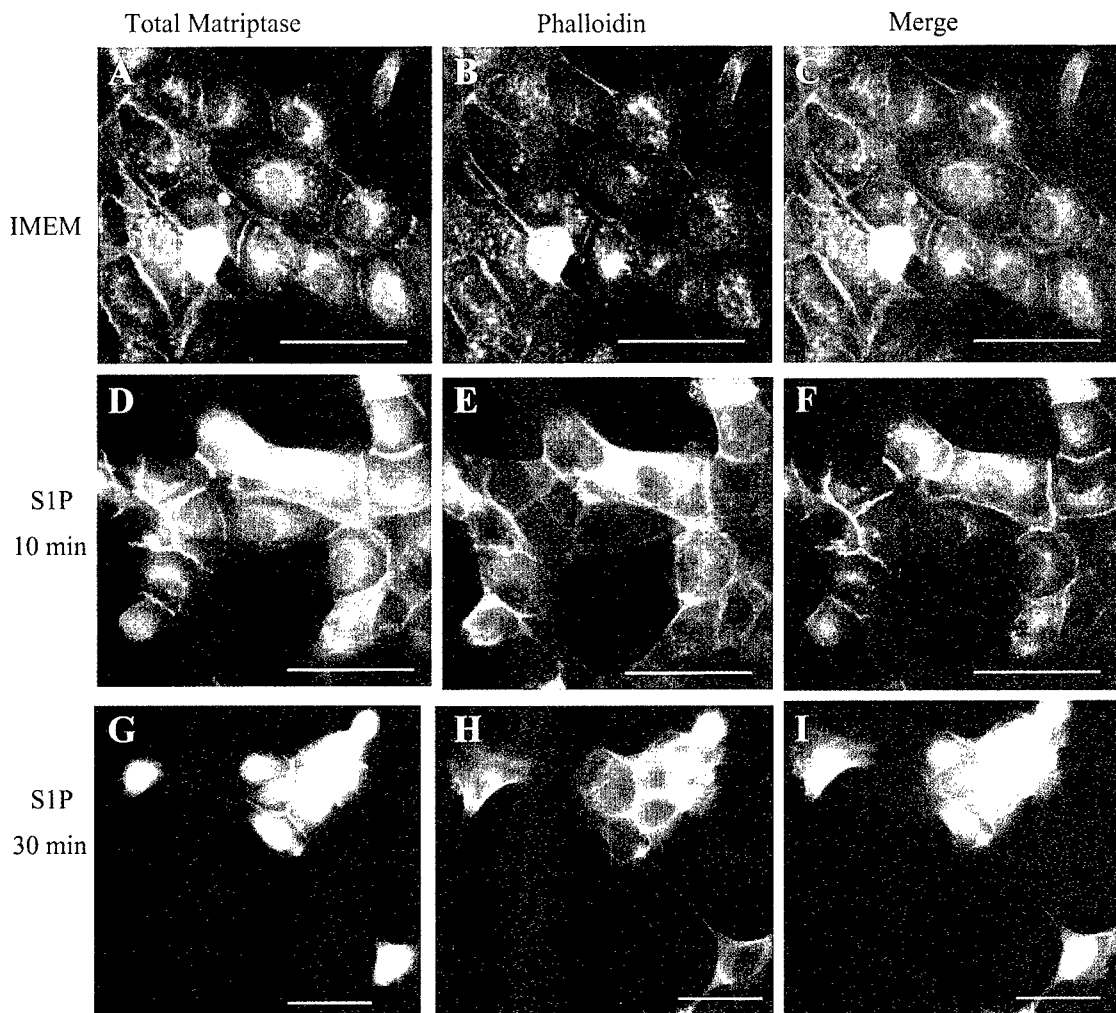


Fig. 1. Sphingosine 1-phosphate (SIP) induces translocation of matriptase to sites of cell-cell contact. Serum-starved 184 A1N4 cells were stimulated with Iscove's minimal essential medium (IMEM) (A–C) or SIP (50 ng/ml) for 10 (D–F) or 30 (G–I) min. Cells were stained for actin with Texas red-conjugated phalloidin (B, E, and H; red), for total matriptase with Alexa Fluor 488-conjugated monoclonal antibody (MAb) M32 (A, D, and G; green), and for nuclei with 4',6-diamidino-2-phenylindole (DAPI; blue). C, E, and I are merged images of A with B, D with E, and G with H, respectively. I shows that matriptase was not detected in the cell periphery of an isolated cell (top left corner) and that matriptase was seen at a small contact area where a long cell process from 1 cell touched at another cell in a cell cluster. Bars, 25 μm .

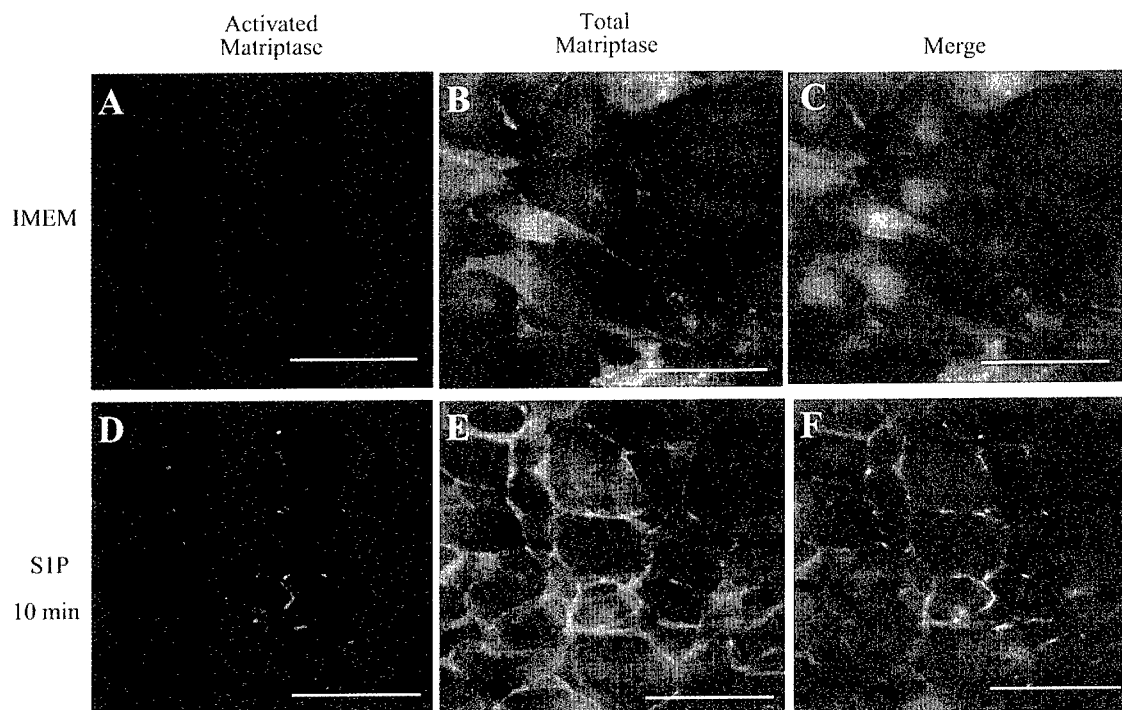


Fig. 2. The onset of matriptase activation induced by SIP. Serum-starved 184 A1N4 cells were stimulated with IMEM (A–C) or SIP (50 ng/ml) for 10 min (D–F). Cells were stained for total matriptase with Alexa Fluor 594-conjugated MAb M32 (B and E; red), for activated matriptase with Alexa Fluor 488-conjugated MAb M69 (A and D; green), and for nuclei with DAPI (blue). C and F are merged images of A with B and D with E, respectively. Bars, 25 μ m.

contact sites on cell peripheries touched by long cellular processes of other cells (Fig. 1, G and D). These results suggest that cell-cell contact may be required for matriptase to stably reside on the cell surface. A parallel change in actin cytoskeletal structure was also observed in response to SIP treatment (Figs. 1, B, E, and H, and 3, B and E). Formation and accumulation of actin filaments were observed at cell peripheries, mainly at cell-cell contacts. The localization of matriptase at cell-cell contacts closely coincided with that of F-actin (Fig. 1, C, F, and I).

Kinetics of matriptase activation at cell-cell contacts. We further examined the appearance and the localization of activated matriptase relative to total matriptase (Fig. 2) and to F-actin (Fig. 3) in 184 A1N4 cells at different time intervals after exposure to SIP. After growth in 0.5% serum for 2 days, 184 A1N4 cells do not express activated matriptase (2–4). Immunofluorescence using MAb M69 that recognizes two-chain matriptase showed only nonspecific staining, with weak, diffuse staining in the cytosol (Fig. 2A), as seen in the control with FITC-labeled secondary antibody alone (data not shown). Ten minutes after exposure to SIP, activated matriptase was detected mainly as tiny spots (Fig. 2D) and the total matriptase accumulated at cell-cell contacts (Fig. 2E). When both images were merged, activated matriptase was clearly colocalized with total matriptase at cell-cell contacts. At 20 and 30 min, activated matriptase was detected more brightly and in elongated patterns (Fig. 3, A and D). When the localization of activated matriptase was compared with the newly formed F-actin at the cell peripheries, activated matriptase closely coincided with F-actin at cell-cell contacts (Fig. 3, C and F). We also noted that subcortical actin belts were not homogeneously formed

around the periphery of cells. More F-actin accumulated at the edges of cell-cell contacts (Fig. 3, B and E). Interestingly, activated matriptase was detected right at those areas where F-actin accumulated the most (Fig. 3, C and F). At 30 min, the staining of activated matriptase elongated along cell-cell contacts (Fig. 3D) and more F-actin accumulated at the cell peripheries (Fig. 3E). Additionally, activated matriptase, like total matriptase, was only detected at sites of cell-cell contacts, for example, in cell clusters (Figs. 2 and 3) or between two cells (Fig. 3G).

SIP induces assembly of adherens junctions in mammary epithelial cells. Because formation of subcortical actin belts at adherens junctions is induced by E-cadherin-mediated cell-cell contact formation (1), and because the role of SIP in adherens junction assembly has been well documented in endothelial cells (12), we further investigated whether SIP also can induce adherens junction assembly in mammary epithelial cells and whether the SIP-induced accumulation of matriptase at cell-cell contacts depends on adherens junction assembly. Given that extracellular Ca^{2+} is required for the homotypic binding between cadherin molecules on adjacent cells, we modulated the availability of extracellular Ca^{2+} , in combination with SIP, to investigate whether SIP can induce assembly of adherens junctions. 184 A1N4 cells were first cultured in regular, calcium-containing (1.8 mM) medium supplemented with 0.5% serum for 2 days. After the cells were switched to calcium-free medium for 30 min, E-cadherin was detected as a diffuse pattern in cytosol, and the subcortical actin belt was not observed (Fig. 4A). When extracellular Ca^{2+} was increased to 1,000 μ M, in the absence of SIP, the subcortical actin belt and E-cadherin

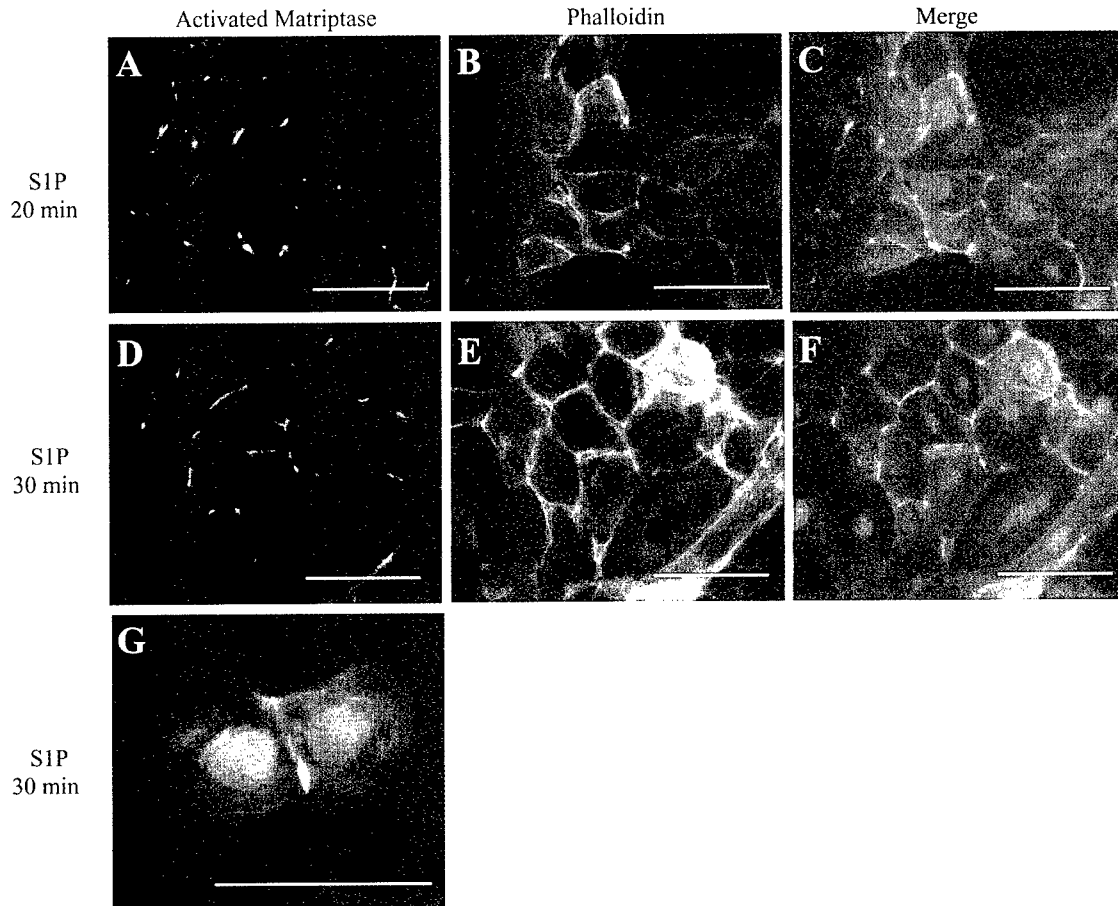


Fig. 3. S1P induces activation of matriptase at cell-cell contacts. Serum-starved 184 AIN4 cells were stimulated with S1P (50 ng/ml) for 20 (A–C) or 30 (D–G) min. Cells were stained for actin with Texas red-conjugated phalloidin (B and E; red) and for activated matriptase with Alexa Fluor 488-conjugated MAb M69 (A and D; green). C and F are merged images of A with B and D with panel E, respectively. G, a merged image of F-actin (red) and activated matriptase (green, but appearing yellow because of the merge), shows that activated matriptase was detected only at contact sites but not on the cellular peripheries without contact with other cells. Bars, 25 μ m.

began to appear at the sites of cell-cell contact, but at very low levels (Fig. 4A). We next exposed these cells to S1P for 30 min. In the absence of extracellular Ca^{2+} , S1P caused some rounding of cells (Fig. 4B). Likewise, there was no translocation of E-cadherin to cell-cell contacts or formation of subcortical actin belts (Fig. 4B). When extracellular Ca^{2+} was increased to 100 and 1,000 μ M, S1P induced to a greater extent translocation of E-cadherin to cell-cell contacts and the formation of subcortical actin belts (Fig. 4B). Although low levels of E-cadherin were seen at the cell-cell contacts when 184 AIN4 cells were grown in medium containing adequate extracellular Ca^{2+} , in the absence of S1P, this cell-cell adhesion E-cadherin failed to resist the extraction of cells with a cytoskeletal stabilizing buffer containing 0.5% Triton X-100 (Fig. 5). In contrast, S1P induced assembly of strong adherens junctions, which were resistant to a wash with 0.5% Triton X-100 (Fig. 5). These data suggest that S1P can induce adherens junction assembly in mammary epithelial cells, as it does in endothelial cells. In contrast to E-cadherin, although matriptase accumulated at cell-cell contacts in response to S1P treatment, the protease was washed away by 0.5% Triton X-100 (Fig. 5). These observations suggest that although matriptase

localization was coincident with that of E-cadherin, at cell-cell contacts the protease may not be incorporated into the tightly bound E-cadherin plaques.

Adherens junction assembly and subcortical actin belt formation are required for matriptase accumulation and activation at cell-cell contacts. The concurrence of matriptase accumulation and activation at cell-cell contacts, adherens junction assembly, and subcortical actin belt formation suggests a potential functional relationship between these S1P-induced events. Therefore, we investigated further whether prevention of subcortical actin belt formation by disruption of F-actin polymerization and prevention of adherens junction assembly by prevention of homotypic binding of E-cadherin each affect the accumulation and activation of matriptase. The pharmacological agents latrunculin B and cytochalasin D (which can inhibit actin polymerization and disrupt microfilament organization) abolished S1P-induced activation of matriptase (Fig. 6). Both latrunculin B (Fig. 6, lanes 2 and 3) and cytochalasin D (Fig. 6, lane 7), but not nocodazole (Fig. 6, lane 5), an inhibitor of microtubule polymerization, completely inhibited S1P-induced matriptase activation. Immunofluorescent staining further revealed that cytochalasin D (Fig. 7) and latrunculin B (data

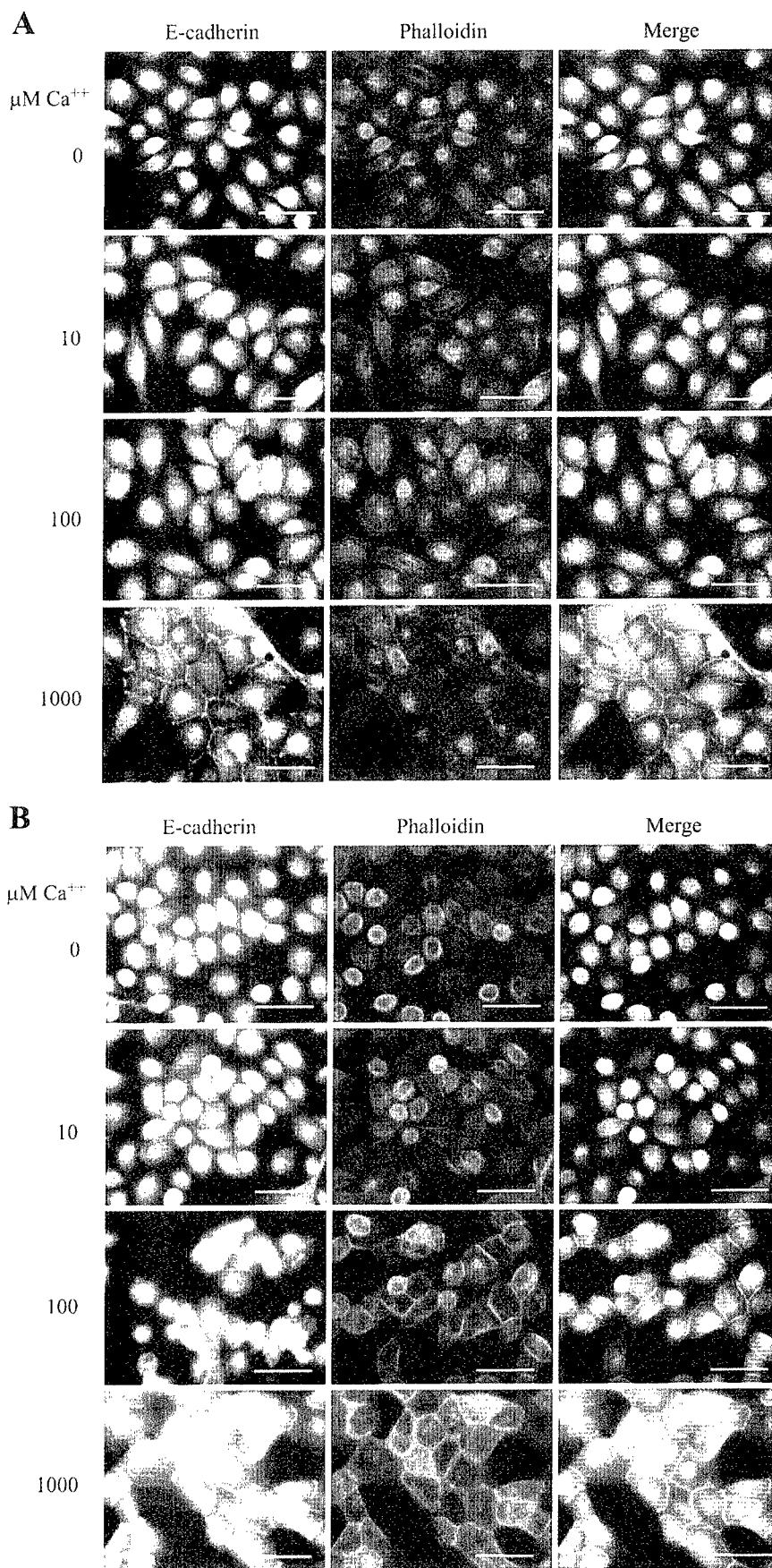


Fig. 4. SIP induces assembly of adherens junctions in mammary epithelial cells. Serum-starved 184 A1N4 cells were preincubated with Ca^{2+} -free DMEM for 30 min, followed by addition of different concentrations of Ca^{2+} as indicated. After 30 min, these cells were then incubated in the presence (B) or absence (A) of 50 ng/ml SIP for 30 min. Cells were stained for E-cadherin with anti-E-cadherin MAb and then FITC-conjugated anti-mouse IgG (green), for F-actin with Texas red-conjugated phalloidin (red), and for nuclei with DAPI (blue). Bars, 25 μm .

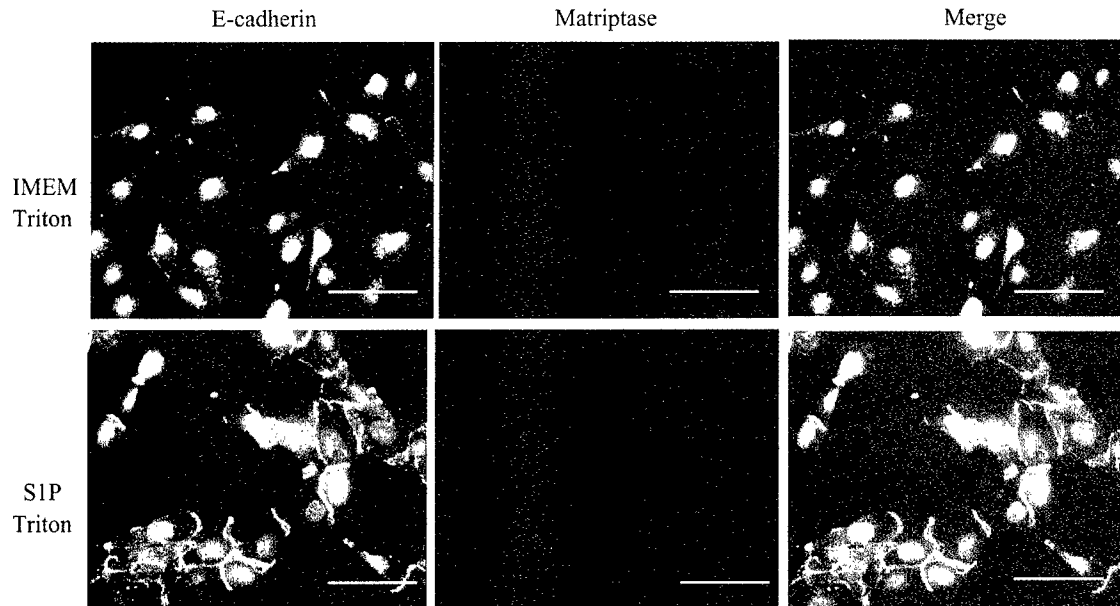


Fig. 5. E-cadherin, but not matriptase, at adherens junctions resists extraction with Triton X-100. Serum-starved 184 AIN4 cells were stimulated with IMEM (A–C) or SIP (50 ng/ml) for 30 min (D–F). Cells were extracted by cytoskeletal stabilizing buffer (in mM: 10 HEPES, 250 sucrose, 150 KCl, 1 EGTA, and 3 MgCl₂ with 0.5% Triton X-100, pH 7.4) on ice for 10 min. Cells were stained for E-cadherin with anti-E-cadherin MAb and then FITC-conjugated anti-mouse IgG (green), for total matriptase with Alexa Fluor 594-conjugated MAb M32 (red), and for nuclei with DAPI (blue).

not shown) prevented not only formation of subcortical actin belt and assembly of adherens junction but also accumulation of matriptase to cell peripheries.

We further modulated the availability of extracellular Ca²⁺, in combination with SIP, to examine the effects of adherens junction assembly on matriptase accumulation at cell-cell contacts. When 184 AIN4 cells were cultured in medium containing 10 μ M Ca²⁺, SIP failed to induce translocation and accumulation of matriptase at cell-cell contacts or β -catenin, an adherens junction marker protein (Fig. 8). When extracellular Ca²⁺ was increased 1,000 μ M, in the absence of SIP, low levels of matriptase and β -catenin were observed at cell-cell contacts. When these cells were exposed to SIP, both matriptase and β -catenin significantly accumulated at cell-cell contacts (Fig. 8). In addition to preventing the accumulation of matriptase at cell-cell contacts, removal of extracellular Ca²⁺ was shown to abrogate SIP-induced matriptase activation (4).

These data suggest that SIP-induced assembly of adherens junctions is required for SIP-induced accumulation and activation of matriptase at cell-cell contacts.

MAb M32, which is directed against matriptase LDLR class A domain III, inhibits SIP-induced matriptase activation but not SIP-induced actin cytoskeletal rearrangement. In our previous study (23), the intact LDLR class A domains of matriptase were shown to be required for matriptase activation in breast cancer cells. This observation is consistent with the hypothesis that matriptase activation is carried out by transactivation, which could require complex protein-protein interactions among matriptase zymogens and other unidentified molecules. Alterations at the calcium cages in LDLR class A domains of matriptase by site-directed point mutation abolished matriptase activation in breast cancer cells (23). Interestingly, this alteration in LDLR class A domain III also destroyed the epitope recognized by the anti-matriptase MAb

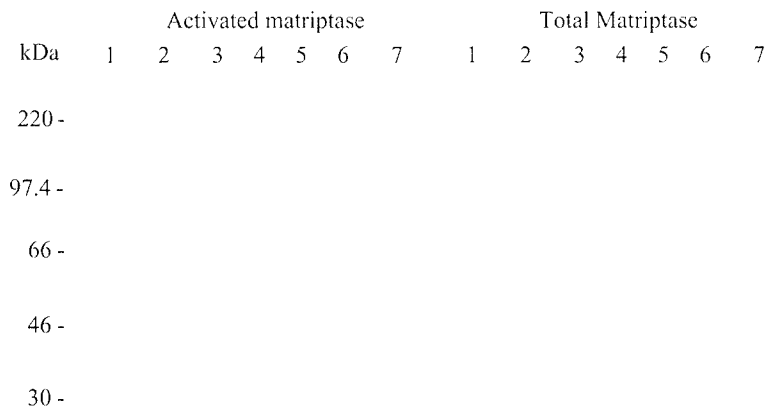


Fig. 6. Latrunculin B and cytochalasin D impair SIP-induced activation of matriptase. Serum-starved 184 AIN4 cells were pretreated for 30 min with the DMSO vehicle (lane 1), latrunculin B (1 μ M, lane 2; 0.1 μ M, lane 3; 0.01 μ M, lane 4), 10 μ M nocodazole (lane 5), or cytochalasin D (0.01 μ M, lane 6; 0.1 μ M, lane 7) and then stimulated for 30 min with 50 ng/ml SIP. Equal amounts of total cell lysates were analyzed by Western blotting with MAb M69, directed against activated matriptase, or MAb M32, directed against total matriptase. The activated matriptase was detected in its noncomplexed form.

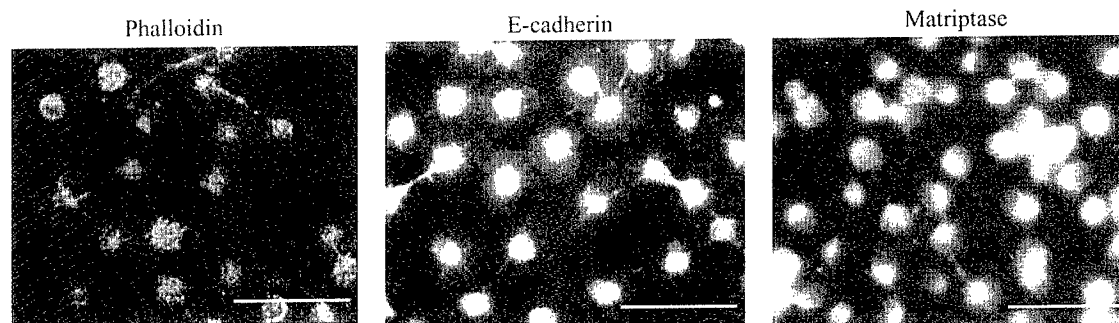


Fig. 7. Translocation of E-cadherin and matriptase by SIP is disrupted by cytochalasin D. Serum-starved 184 A1N4 cells were pretreated for 30 min with 1 μ M cytochalasin D and then stimulated for 30 min with 50 ng/ml SIP. Cells were stained for F-actin with Texas red-conjugated phalloidin (red), for E-cadherin with anti-E-cadherin MAb and then FITC-conjugated anti-mouse IgG (green), and for total matriptase with Alexa Fluor 488-conjugated MAb M32 (green). Bars, 25 μ m.

M32 (Fig. 9). Therefore, we tested further whether this anti-matriptase MAb could inhibit SIP-induced matriptase activation and SIP-induced actin cytoskeletal rearrangement in immortalized mammary epithelial cells. Pretreatment of 184 A1N4 cells with MAb M32 for 1 h clearly showed inhibition of SIP-induced matriptase activation (Fig. 10G). This inhibition of SIP-induced matriptase activation by MAb M32 could result from the potential inhibitory activity of matriptase trans-activation. Alternatively, pretreatment of MAb M32, which could bind to the matriptase on the cell surfaces before SIP

treatment (Figs. 1A, 2B, and 8), could cause internalization of matriptase and thus may interfere with its later accumulation induced by SIP treatment, because matriptase was seen as punctate after SIP treatment (Fig. 10D). In contrast to the inhibition of matriptase activation, anti-matriptase MAb M32 did not affect the formation of subcortical actin belts (Fig. 10, E and H). These results suggest that although actin cytoskeletal rearrangement is required for SIP-induced matriptase activation, matriptase activity is not important for SIP-induced actin cytoskeletal rearrangement.

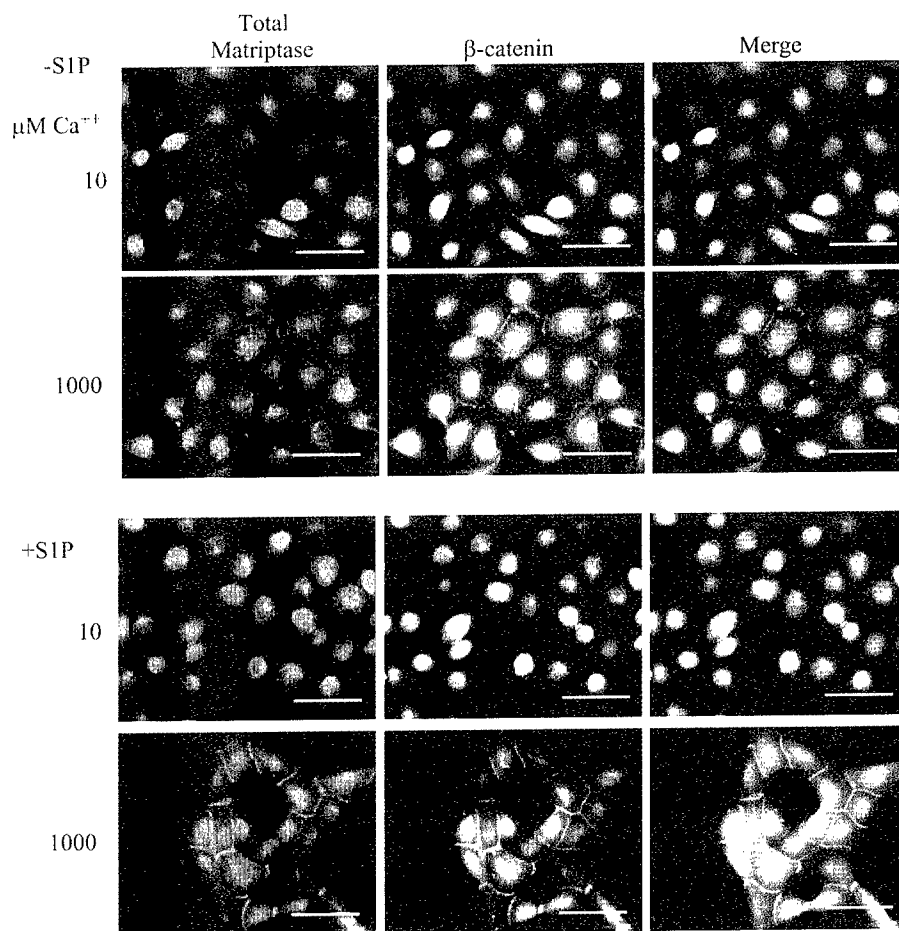


Fig. 8. SIP-induced translocation and accumulation of matriptase at cell-cell contacts depends on assembly of adherens junctions. Serum-starved 184 A1N4 cells were preincubated with Ca^{2+} -free DMEM for 30 min, followed by addition of 10 and 1,000 μ M Ca^{2+} , as indicated. After 30 min, these cells were then incubated in the absence (–) or presence (+) of 50 ng/ml SIP for 30 min. Cells were stained for total matriptase with Alexa Fluor 549-conjugated MAb M32 (red), for β -catenin with FITC-conjugated anti- β -catenin MAb (green), and for nuclei with DAPI (blue). Bars, 25 μ m.

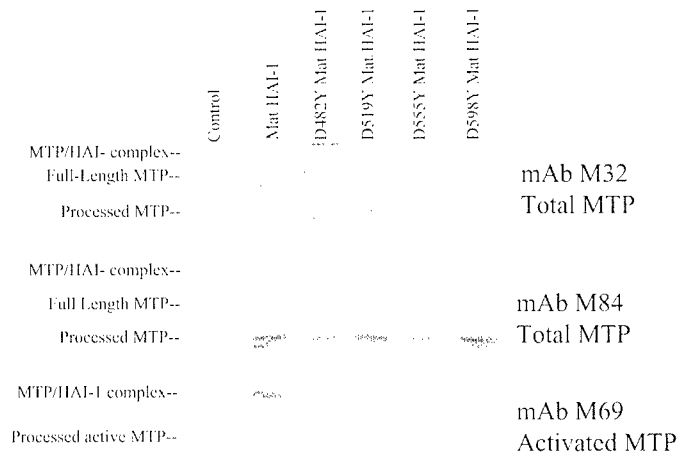


Fig. 9. Epitope mapping for anti-matriptase MAb M32. BT549 breast cancer cells were transfected with control pcDNA3.1 vector (Control), both matriptase and hepatocyte growth factor (HGF) activator inhibitor 1 (HAI-1) cDNAs simultaneously (Mat/HAI-1), or with matriptase containing mutations in the low-density lipoprotein (LDL) receptor class A domain (D482Y Mat, D519Y Mat, D555Y Mat, D598Y Mat) together with HAI-1 as indicated. Total matriptase was detected by Western blotting with MAb M32 (*top*) and MAb M84 (*middle*), both of which recognize total (latent + activated) matriptase. Both MABs recognize the 120-kDa matriptase-HAI-1 complex, the 70-kDa processed form, and the 95-kDa full-length matriptase. The 95-kDa full-length matriptase was detected at a relatively higher level in the forced expression system but was not detected or was detected at a very low level in those cells that endogenously express matriptase. The epitope recognized by MAb M32 was destroyed in D555Y matriptase. Activated matriptase was detected by using MAb M69, mainly as a 120-kDa matriptase-HAI-1 complex (*bottom*).

DISCUSSION

In this study we describe a series of SIP-induced cellular events leading to matriptase activation in immortalized mammary epithelial cells. Our findings indicate that, rather than being directly engaged and activated around the peripheries of cells and within cells, matriptase accumulates at cell-cell contacts soon after exposure to SIP. The onset of matriptase activation occurs at small areas of cell-cell contacts and then extends along cell-cell contacts. Accumulation of latent matriptase at cell-cell contacts occurs in response to SIP treatment and appears to be prerequisite for matriptase activation. This accumulation of matriptase at cell-cell contacts could result from movement of intracellular and perijunctional matriptase. In response to SIP, assembly of E-cadherin-mediated cell-cell adhesion and formation of subcortical actin belt are required for matriptase translocation to and accumulation at cell-cell contacts and its subsequent activation. The coupling of matriptase activation to actin cytoskeletal rearrangement and formation of stable adherens junctions may ensure the restriction of the proteolytic activity of matriptase to cell-cell contacts. Thus the cell-cell contacts could be the functional location of matriptase in immortal mammary epithelial cells.

The identification of cell-cell contacts as the functional location of matriptase in immortalized mammary epithelial cells is in contrast to its localization in breast cancer cells. In T-47D breast cancer cells, matriptase was detected at cell peripheries (17), as in 184 A1N4 mammary epithelial cells. However, in T-47D cells the protease was detected on cell surfaces in isolated breast cancer cells as well as on outer cell surfaces in cell clusters (17). Notably, in cancer cells, the

protease is concentrated on membrane ruffles, suggesting that T-47D breast cancer cells could convert a physiological "cell junction protease" to a potential cancer "invasion protease." Matriptase, expressed in cancer cells, could recruit and activate uPA, an important extracellular matrix-degrading protease, and HGF, a prominent cell motility factor, to invasive cell edges (14, 26). Furthermore, the accumulation of activated matriptase on membrane ruffles was significantly increased by epidermal growth factor (EGF) (3). Besides its role in growth regulation, EGF has been implicated in cell motility and potentially in cancer invasion and metastasis (30). In MCF-7 breast cancer cells, the activated matriptase was also detected within cells, suggesting an intracellular activation or an active internalization of the protease. Furthermore, in contrast to immortalized mammary epithelial cells, which depend on SIP for matriptase activation, breast cancer cells have developed an autonomous mechanism to constitutively activate matriptase regardless of the presence of SIP (3). Therefore, breast cancer cells could deregulate matriptase, with respect to both its subcellular localization and its activation.

In our previous study (23), we showed that activation of matriptase is likely to be carried out by transactivation, an unconventional mechanism for serine protease activation whereby a matriptase zymogen molecule could interact with and cleave (activate) another matriptase zymogen molecule. We have proposed that transactivation could occur in an activation complex that contains matriptase zymogen molecules and other yet unidentified proteins. Because the LDL receptor class A domains and *N*-glycosylation of the protease at its CUB domain and serine protease domain are required for its activation, these structural requirements for matriptase activation could result from the protein-protein interactions in this proposed activation complex (23). However, how the transactivation is regulated in immortalized mammary epithelial cells and why breast cancer cells constitutively activate matriptase remain largely unknown. In this study, we have shown that SIP induces accumulation of matriptase at cell-cell contacts (Fig. 1) and that activation of matriptase may begin at tiny areas of cell-cell contact, presumably signifying activation centers (Fig. 2). Activation then spreads along cell-cell contacts (Fig. 3). It is likely that SIP could also translocate other required components for matriptase transactivation to those activation centers, for the onset of matriptase activation. After initial activation of matriptase, there may be two possible mechanisms for its further activation: transactivation and activation of matriptase by newly active matriptase along the cell-cell contacts, where matriptase zymogen has accumulated.

Among the SIP downstream effectors, matriptase may be unique. The functional locations of other SIP downstream effectors are either in the cytosol, as for adenylate cyclase and ERK2 MAP kinase, or on the cytoplasmic face of the plasma membrane, as for Rho family GTPases. The bulk of matriptase molecules, including their serine protease domains, are located at the exoplasmic face of the plasma membrane (17). Therefore, matriptase could act on its substrates at the extracellular face of cell-cell junctions. Three protein substrates of matriptase *in vitro* have been identified to date. These are HGF, uPA, and the protease-activated receptor-2 (PAR-2) (14, 26). List et al. (18) suggested that the defects in epidermal barrier function, hair follicle de-

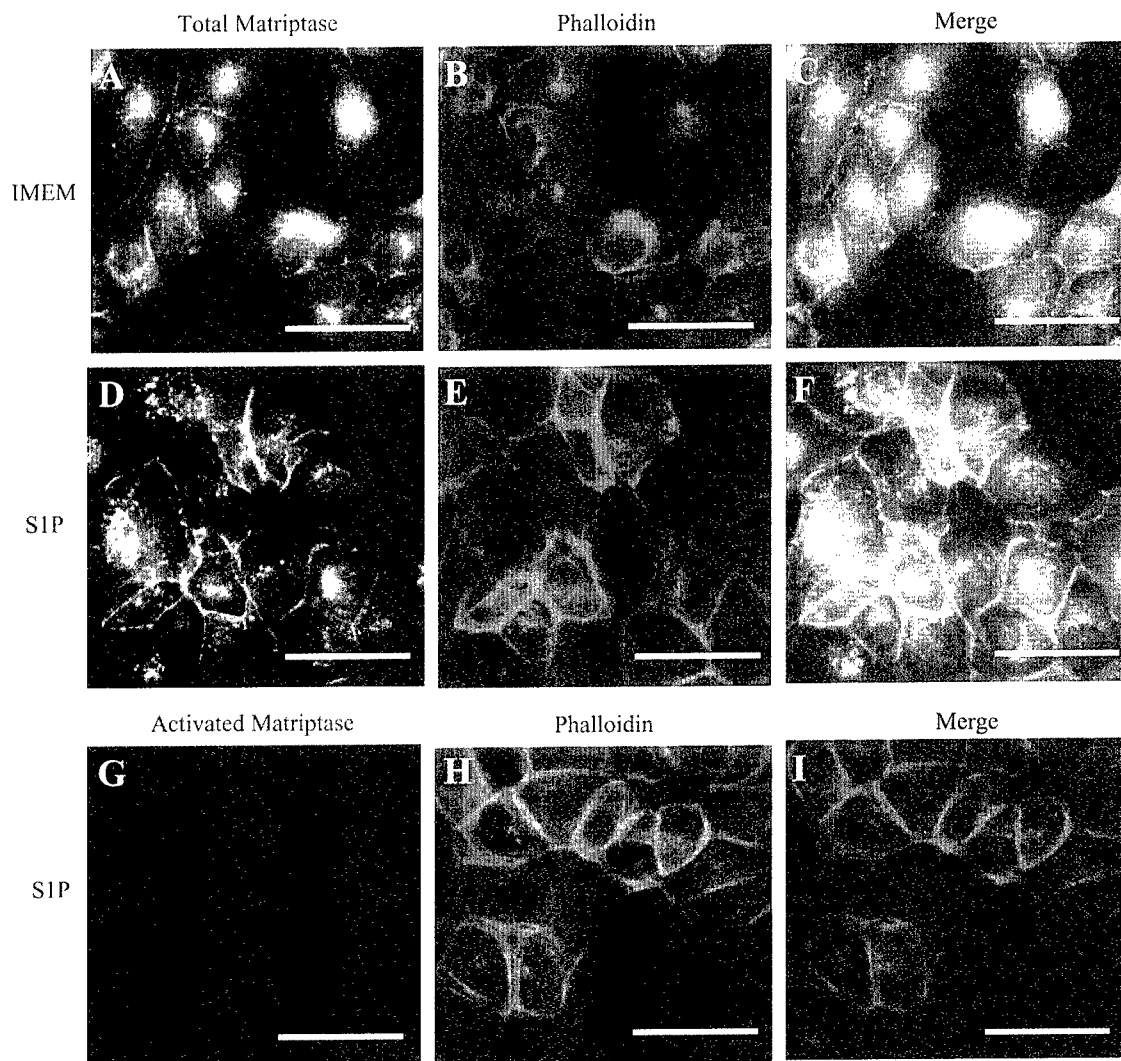


Fig. 10. Anti-matriptase MAb M32 does not suppress SIP-induced actin cytoskeletal rearrangement. Serum-starved 184 A1N4 cells were incubated with 5 μ g/ml anti-matriptase MAb M32 for 1 h and then stimulated with IMEM (A–C) or SIP (D–F) for 30 min. Cells were stained for total matriptase with FITC-conjugated anti-mouse IgG (A and D; green), for activated matriptase with Alexa Fluor 488-conjugated M69 (G), for actin with Texas red-conjugated phalloidin (B, E, and H; red), and for nuclei with DAPI (blue). C, E, and I are merged images of A with B, D with E, and G with H, respectively. Bars, 25 μ m.

velopment, and thymic homeostasis in matriptase-deficient mice are not likely to result from the lack of activation of uPA. Although matriptase has been proposed to activate uPA in human breast cancer, where uPA is overexpressed, it remains to be further determined whether matriptase could activate uPA at cell-cell contacts in physiological settings. HGF, a secreted cell growth/motility factor, has pleiotropic functions, including direct control of cell-cell and cell-substrate adhesion in epithelia. The HGF membrane receptor c-Met is expressed at cell-cell contacts of epithelial cells (6). Thus matriptase could serve as an activator for HGF at the cell-cell contacts where the c-Met receptor is located. PAR-2, a seven-transmembrane G protein-coupled receptor, has been identified in a number of epithelial tissues and is likely to be located at the basolateral surface of cells (5, 7). Matriptase could cleave within the extracellular amino terminus of PAR-2 to release a tethered ligand that binds to and activates the cleaved receptor. Therefore, matriptase could serve as a unique SIP effector that can transduce signals

from G protein-coupled receptors to growth factor receptors or other G protein-coupled receptors.

ACKNOWLEDGMENTS

We thank Drs. Peter Burbello and Steve Byers, Lombardi Cancer Center, Georgetown University, for helpful discussions.

Present addresses: R.-J. Hung, I.-W. J. Hsu, and M.-J. Lee, Dept. of Life Science, National Tsing-Hua University, 101, Section 2 Kuang Fu Road, Hsinchu 300, Taiwan; M. D. Oberst, National Cancer Institute, National Institutes of Health, Building 10 Room 3B47, 9000 Rockville Pike, Bethesda, MD 20892.

GRANTS

This study was supported by a grant from Corvas International, Inc., National Cancer Institute (NCI) Grant P50-CA0-58185, and US Department of Defense (DOD) Grant DAMD 17-02-1-0391. Salary support for C.-Y. Lin was also provided by US DOD Grant DAMD 17-01-1-252 and by Susan G. Komen Breast Cancer Foundation Grant BCTR0100345. This work was supported in part by the Lombardi Cancer Center Microscopy and Imaging Shared Resource, NCI Grant 2P30-CA-51008.

REFERENCES

- Adams CL, Chen YT, Smith SJ, and Nelson WJ. Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. *J Cell Biol* 142: 1105–1119, 1998.
- Benaud C, Dickson RB, and Lin CY. Regulation of the activity of matriptase on epithelial cell surfaces by a blood-derived factor. *Eur J Biochem* 268: 1439–1447, 2001.
- Benaud CM, Oberst M, Dickson RB, and Lin CY. Deregulated activation of matriptase in breast cancer cells. *Clin Exp Metastasis* 19: 639–649, 2002.
- Benaud C, Oberst M, Hobson JP, Spiegel S, Dickson RB, and Lin CY. Sphingosine 1-phosphate, present in serum-derived lipoproteins, activates matriptase. *J Biol Chem* 277: 10539–10546, 2002.
- Bertog M, Letz B, Kong W, Steinhoff M, Higgins MA, Bielfeld-Ackermann A, Fromter E, Bunnett NW, and Korbmacher C. Basolateral proteinase-activated receptor (PAR-2) induces chloride secretion in M-1 mouse renal cortical collecting duct cells. *J Physiol* 521: 3–17, 1999.
- Crepaldi T, Pollack AL, Prat M, Zborek A, Mostov K, and Comoglio PM. Targeting of the SF/HGF receptor to the basolateral domain of polarized epithelial cells. *J Cell Biol* 125: 313–320, 1994.
- Danahay H, Withey L, Poll CT, van de Graaf SF, and Bridges RJ. Protease-activated receptor-2-mediated inhibition of ion transport in human bronchial epithelial cells. *Am J Physiol Cell Physiol* 280: C1455–C1464, 2001.
- Hooper JD, Clements JA, Quigley JP, and Antalis TM. Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes. *J Biol Chem* 276: 857–860, 2001.
- Kang JY, Dolled-Filhart M, Ocal IT, Singh B, Lin CY, Dickson RB, Rimm DL, and Camp RL. Tissue microarray analysis of hepatocyte growth factor/Met pathway components reveals a role for Met, matriptase, and hepatocyte growth factor activator inhibitor 1 in the progression of node-negative breast cancer. *Cancer Res* 63: 1101–1105, 2003.
- Kim MG, Chen C, Lyu MS, Cho EG, Park D, Kozak C, and Schwartz RH. Cloning and chromosomal mapping of a gene isolated from thymic stromal cells encoding a new mouse type II membrane serine protease, epithin, containing four LDL receptor modules and two CUB domains. *Immunogenetics* 49: 420–428, 1999.
- Kon J, Sato K, Watanabe T, Tomura H, Kuwabara A, Kimura T, Tamama K, Ishizuka T, Murata N, Kanda T, Kobayashi I, Ohta H, Ui M, and Okajima F. Comparison of intrinsic activities of the putative sphingosine 1-phosphate receptor subtypes to regulate several signaling pathways in their cDNA-transfected Chinese hamster ovary cells. *J Biol Chem* 274: 23940–23947, 1999.
- Lee MJ, Thangada S, Claffey KP, Ancellin N, Liu CH, Kluk M, Volpi M, Sha'afi RI, and Hla T. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* 99: 301–312, 1999.
- Lee MJ, Van Brocklyn JR, Thangada S, Liu CH, Hand AR, Menzelev R, Spiegel S, and Hla T. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science* 279: 1552–1555, 1998.
- Lee SL, Dickson RB, and Lin CY. Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. *J Biol Chem* 275: 36720–36725, 2000.
- Lin CY, Anders J, Johnson M, and Dickson RB. Purification and characterization of a complex containing matriptase and a Kunitz-type serine protease inhibitor from human milk. *J Biol Chem* 274: 18237–18242, 1999.
- Lin CY, Anders J, Johnson M, Sang QA, and Dickson RB. Molecular cloning of cDNA for matriptase, a matrix-degrading serine protease with trypsin-like activity. *J Biol Chem* 274: 18231–18236, 1999.
- Lin CY, Wang JK, Torri J, Dou L, Sang QA, and Dickson RB. Characterization of a novel, membrane-bound, 80-kDa matrix-degrading protease from human breast cancer cells. Monoclonal antibody production, isolation, and localization. *J Biol Chem* 272: 9147–9152, 1997.
- List K, Haudenschild CC, Szabo R, Chen W, Wahl SM, Swaim W, Engelholm LH, Behrendt N, and Bugge TH. Matriptase/MT-SP1 is required for postnatal survival, epidermal barrier function, hair follicle development, and thymic homeostasis. *Oncogene* 21: 3765–3779, 2002.
- Netzel-Arnett S, Hooper JD, Szabo R, Madison EL, Quigley JP, Bugge TH, and Antalis TM. Membrane anchored serine proteases: a rapidly expanding group of cell surface proteolytic enzymes with potential roles in cancer. *Cancer Metastasis Rev* 22: 237–258, 2003.
- Oberst M, Anders J, Xie B, Singh B, Ossandon M, Johnson M, Dickson RB, and Lin CY. Matriptase and HAI-1 are expressed by normal and malignant epithelial cells in vitro and in vivo. *Am J Pathol* 158: 1301–1311, 2001.
- Oberst MD, Johnson MD, Dickson RB, Lin CY, Singh B, Stewart M, Williams A, al Nafussi A, Smyth JF, Gabra H, and Sellar GC. Expression of the serine protease matriptase and its inhibitor HAI-1 in epithelial ovarian cancer: correlation with clinical outcome and tumor clinicopathological parameters. *Clin Cancer Res* 8: 1101–1107, 2002.
- Oberst MD, Singh B, Ossandon M, Dickson RB, Johnson MD, and Lin CY. Characterization of matriptase expression in normal human tissues. *J Histochem Cytochem* 51: 1017–1025, 2003.
- Oberst MD, Williams CA, Dickson RB, Johnson MD, and Lin CY. The activation of matriptase requires its noncatalytic domains, serine protease domain, and its cognate inhibitor. *J Biol Chem* 278: 26773–26779, 2003.
- Okamoto H, Takuwa N, Gonda K, Okazaki H, Chang K, Yatomi Y, Shigematsu H, and Takuwa Y. EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a $G_{i/o}$ to multiple signaling pathways, including phospholipase C activation, Ca^{2+} mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J Biol Chem* 273: 27104–27110, 1998.
- Sun J, Pons J, and Craik CS. Potent and selective inhibition of membrane-type serine protease 1 by human single-chain antibodies. *Biochemistry* 42: 892–900, 2003.
- Takeuchi T, Harris JL, Huang W, Yan KW, Coughlin SR, and Craik CS. Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J Biol Chem* 275: 26333–26342, 2000.
- Takeuchi T, Shuman MA, and Craik CS. Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. *Proc Natl Acad Sci USA* 96: 11054–11061, 1999.
- Tanimoto H, Underwood LJ, Wang Y, Shigemasa K, Parmley TH, and O'Brien TJ. Ovarian tumor cells express a transmembrane serine protease: a potential candidate for early diagnosis and therapeutic intervention. *Tumour Biol* 22: 104–114, 2001.
- Van Brocklyn JR, Tu Z, Edsall LC, Schmidt RR, and Spiegel S. Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. *J Biol Chem* 274: 4626–4632, 1999.
- Wells A. Tumor invasion: role of growth factor-induced cell motility. *Adv Cancer Res* 78: 31–101, 2000.
- Yamazaki Y, Kon J, Sato K, Tomura H, Sato M, Yoneya T, Okazaki H, Okajima F, and Ohta H. Edg-6 as a putative sphingosine 1-phosphate receptor coupling to Ca^{2+} signaling pathway. *Biochem Biophys Res Commun* 268: 583–589, 2000.
- Zhang Y, Cai X, Schlegelberger B, and Zheng S. Assignment1 of human putative tumor suppressor genes ST13 (alias SNC6) and ST14 (alias SNC19) to human chromosome bands 22q13 and 11q24→q25 by in situ hybridization. *Cytogenet Cell Genet* 83: 56–57, 1998.